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14. ABSTRACT

This report describes studies during the previous 12 months that document the use of mouse models of platelet dysfunction in the progression of cancer to metastatic disease. During the next year we propose to examine the relevance of platelet receptors in models of spontaneous metastasis. A transgenic C57BL/6J mouse colony expressing MMTV-PyV middle antigen in mammary epithelium has been developed. Mammary gland tumor and lung metastases develop in these mice spontaneously so they will be used in spontaneous metastasis experiments. Breeding with congenic colonies with defective platelet GP receptors will determine the relevance of platelet adhesion and activation to spontaneous tumor metastasis. The importance of metastasis in the prognosis for recovery from breast cancer cannot be under emphasized. Indeed, the spread of metastatic disease represents a fundamental change in significantly shortening the life span of patients with breast cancer. Thus, understanding the molecules that regulate metastasis identifies potential targets for therapeutic intervention that could significantly improve the prognosis for the breast cancer patient. Although the proposed studies are basic in their approach, the ability to target platelet receptors in anti-thrombotic therapy (inhibitor of blood clotting) is an ongoing avenue for product development within several large pharmaceutical companies.

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Introduction

The platelet paradigm in hemostasis and thrombosis involves an initiation step dependent upon platelet membrane receptors binding to ligands on a damaged or inflamed vascular surface. Once bound to the surface, platelets provide a unique microenvironment supporting the accumulation of more platelets and the elaboration of a fibrin-rich network produced by coagulation factors. This paradigm has been established from decades of research. The platelet-specific receptor, glycoprotein (GP) Ib-IX, is critical in this process and can initiate the formation of a platelet-rich thrombus by tethering the platelet to a thrombogenic surface. Several ligands binding to GP Ib-IX have been identified, including von Willebrand factor (vWF) and thrombin, illustrating platelet GP Ib-IX as a major initiator of platelet thrombus formation in the arterial circulation. Newer, emerging data supports a role for platelets in pathological events beyond the prevention of blood loss. In our ongoing studies, **we are testing the hypothesis that platelet GP Ib-IX contributes to malignancy** [1]. Knockout and transgenic mouse colonies have been generated in our laboratory and bred to C57BL/6J animals to generate several congenic strains (> 10 generation backcrosses) with dysfunctional platelet GP Ib-IX. Our outlined studies are providing results to test the hypothesis and will provide new information on the link between platelets and cancer. Metastasis is estimated to be the cause of nearly 90% of all human cancer deaths. Long term, the outlined studies will evaluate whether adjunct anti-GP Ib-IX therapy could benefit the breast cancer patient with malignant disease.

Body

Below we list the 3 Specific Aims from our original submission (blue font) followed by specific details (black font) for how each Aim has progressed during the covered date for this report (Sep 1, 2009 – Aug 31, 2010).

Specific Aim 1. To define the temporal sequence of events linking platelet GP Ib-IX and tumorigenesis. This aim will be achieved with an integrated approach of in vivo imaging using reporter tagged cell lines and histological analyses.

The experiments related to this aim were detailed in our previous year's progress report (Sep 1, 2008 – Aug 31, 2009). The outcomes of these experiments are not repeated here because they were not focus of this 12 month reporting period.

Specific Aim 2. To examine the relevance of thrombin binding to GP Ib-IX in tumorigenesis. Thrombin is a central molecule in hemostasis and a common thread for cancer as many tumor cells express thrombin. We have a mouse model with normal human GP Ib-IX/vWF binding but blocked binding between GP Ib-IX and thrombin. Experiments are proposed to determine if a GP Ib-IX/thrombin axis is critical in tumor development.

We developed the mouse Y276F model that represents a single tyrosine to phenylalanine mutation within the extracellular domain of platelet GP Ib (Fig 1). The thrombosis

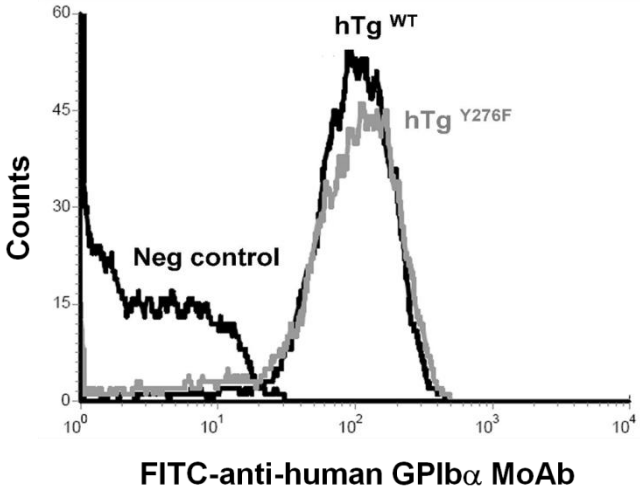

Figure 1. GPIbα expression on the platelet surface. *Surface expression of GPIbα was evaluated using a FITC-labeled anti-human GP Ibα monoclonal antibody. Platelets from hTg^{WT} and hTg^{Y276F} animals express similar levels of GP Ibα on their surface. Nontransgenic or normal mice were used as negative control. A 10 generation backcross to generate a congenic mouse colony has been major focus of effort related to this aim for this reporting period.*

consequences of this mutation have been published but this work was peformed in a mixed genetic strain background comparing littermates from het/het crosses, a situation unsuitable syngenic model of metastasis [2]. To perform the experiments for SA2 required the generation of the Y276F mutation in a syngenic mouse background, i.e, a mouse model where the strain background (C57BL/6J) matches the source of mouse tumor cell lines. In order to achieve this we have been performing extensive mouse husbandry crossing the Y276F to wild-type C57BL/6J mice for 10 generations, the gold-standard in generating a congenic mouse strain. While this may seem trivial, it has been a time consuming (>18 mos.) endeavor to generate the mouse colony that will be suitable for the syngeneic experiment. A similar breeding strategy has been done with the straight knockouts utilized in SA1. We have invested the majority of efforts towards SA3 that are described below, but finishing SA2 will be the major effort for the upcoming reporting period.

Specific Aim 3. To examine the relevance of platelet GP Ib-IX in a model of spontaneous metastasis.

Experimental metastasis (the primary approach for SA1 and SA2) is one of the most commonly used laboratory tools to study the role of various proteins in metastasis [3]. In xenograft models, various immortalized human cancer cells (such as MDA MB 231, MCF-7, etc.) are injected into the tail vein, portal vein, or left cardiac ventricle and monitored for their ability to establish tumor burden in mice. Xenograft models are useful to study the behavior of human cancer cell lines but the absence of a fully functional immune system in the host completely ignores the contribution of the immune system in tumor cell survival. In syngenic models there is a genetic match between the host mouse and the tumor cell allowing tumor cell survival to be monitored in the presence of a functional immune system. In addition, injections of immortalized cancer cell lines have been widely criticized for their indefinite *in vitro* culture

which may have altered cellular properties. As such, experimental metastasis presents a rather artificial model which simplifies the process of metastasis to a single step of dissemination as opposed to an ongoing or continuous dissemination of cells from primary tumors in natural settings of metastasis [3]. However, the large bolus of tumor cells administered in experimental metastasis may reflect events occurring during the late stages of metastasis when a large number of tumor cells are shed from the primary tumor [4]. An alternative to experimental metastasis models are models of spontaneous tumor formation and metastasis. In these models, transgenic mice express an oncogene producing spontaneous primary tumors and metastases recapitulating some aspects of human cancer pathology [5].

In 1992, a mouse model of spontaneous breast cancer, MMTV-PyMT, was reported [6]. A transgenic insertion of the gene encoding the polyoma virus middle T antigen (PyMT) generated rapid and spontaneous multifocal mammary adenocarcinoma as a consequence of PyMT expression. Middle T antigen is a viral oncogene which provides a scaffold on the plasma membrane for constitutive activation of various signaling proteins [7]. Mammary tissue-specific expression is supported by a mouse mammary tumor virus long terminal repeat promoter (MMTV-LTR). The developing primary foci are followed by the appearance of spontaneous metastatic tumors on the lungs in more than 85% of 4-5 month old animals. In PyMT mice, palpable tumors are apparent on mammary glands by 9-10 wks and lung metastasis is detectable by 12-20 wks. Short latency, high penetrance, and a high incidence of lung metastasis occurring independent of pregnancy makes this a useful model to study spontaneous metastasis [8].

Relative to this aim we have made significant progress briefly outlined below. First, we obtained a mouse colony from Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) over-expressing the PyMT antigen [9]. Mice from this colony spontaneously develop primary breast tumors that at a frequency of ~80% will metastasize to the lung. We have generated compound mice expressing the PyMT antigen and also containing our platelet mutation in the receptor complex, GP Ib-IX. We have also just initiated studies characterizing the frequency of metastasis in these compound heterozygous animals. The flow diagram (Fig 4 from the previous year's repor) illustrated our strategy for generating these animals and also the strategy for phenotyping these animals. We point out the breeding strategy itself (just to generate sufficient animals for analysis) has taken ~12 months.

Primary tumor growth in GP1b^{null};PyMT mice

A compound transgenic mouse colony GP1b^{null};PyMT was generated which is devoid of platelet GP Ib and expresses the polyoma virus middle T-antigen (PyMT) under the control of a mouse mammary tumor virus promoter (MMTV) (Fig 2). The PyMT oncogene in mice initiates the spontaneous development of a mammary adenocarcinoma by the age of 8-10 weeks without pregnancy or any other stimuli. To examine if the absence of platelet GP Ibα affects the growth of primary tumors, the primary tumor growth was followed on mammary glands of control GP1b^{WT};PyMT and GP1b^{null};PyMT mice for 20 weeks. Detectable tumors were first apparent on

mammary glands by the age of 9-10 weeks. No difference in latency was found between control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies. The latency period on both the colonies varied from the age of 9-10 weeks to the age of 20 weeks. The expression of PyMT in both groups resulted in mammary adenocarcinomas by the age of 20 wks on single and sometimes multiple sites. The weight of primary tumors at 20 wks of age was more in the animals which developed primary tumors earlier than the animals in which primary tumor developed in later ages. At the age of 20 weeks animals were sacrificed.

Figure 2. GP Ibα expression on the platelets of control GP1b^{WT};PyMT and GP1b^{null};PyMT mice. *Flow cytometry profiles of platelets of control GP1b^{WT};PyMT (black line) and GP1b^{null};PyMT (gray area) generated by a PE conjugated rat anti-mouse CD42b (GP Ibα) monoclonal antibody.*

The cumulative tumor weight at 20 weeks of age ranged from 0.3 g - 5.7 g in control GP1b^{WT};PyMT mice (n=16) and 0.13 g - 7.1 g in GP1b^{null};PyMT mice (n=25). The mean cumulative tumor weight of primary tumors in control GP1b^{WT};PyMT and GP1b^{null};PyMT mice was 1.9 g and 2 g respectively (*p* value = 0.46). These observations suggest growth of mammary adenocarcinoma is not affected by the absence of platelet GP Ibα in the MMTV-PyMT model (Fig 3).

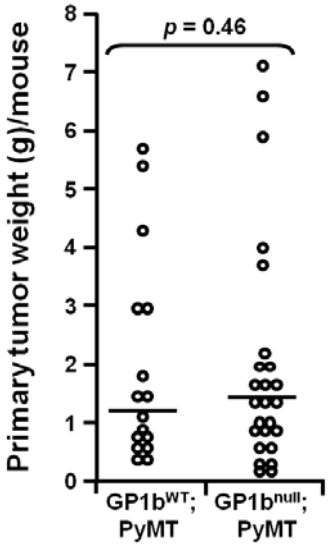

Figure 3. Primary tumor weight in control GP1b^{WT};PyMT and GP1b^{null};PyMT mice. *Nulligravida control GP1b^{WT};PyMT (n = 16) and GP1b^{null};PyMT (n = 25) female mice were sacrificed at the age of 20 wks. All primary tumors from all mammary glands were collected. Dot plot is showing the cumulative primary tumor burden (weight in grams) on mammary glands. Horizontal bar represents the median value. p value (Student's t test) is shown.*

Spontaneous metastasis in GP1b^{null};PyMT mice

To quantitate total metastatic tumor burden within lungs, a real-time PCR assay was performed. The mRNA level for PyMT in lungs was indicative of spontaneous lung metastasis of the mammary adenocarcinoma since PyMT expression was restricted to mammary glands using the mouse mammary tumor virus promoter [10]. G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a reference endogenous control transcript. A comparative C_T (threshold temperature) method was utilized for quantitation using the formula $2^{-\Delta\Delta CT}$. Before running the real-time PCR, efficiencies of primers for PyMT and G3PDH was checked by running validation experiments and found to be approximately equal. cDNA was prepared from PyMT induced primary mammary gland tumors and used as a positive control for the calculations by considering mRNA levels of PyMT as 1.

The extent of spontaneous lung metastasis was evaluated at the age of 20 wks in control GP1b^{WT};PyMT and GP1b^{null};PyMT animals. Mice were divided in two groups on the basis of cumulative primary tumor weight at the time of sacrifice; less than 1.5 g and greater than 1.5 g. In the mice which had cumulative primary tumor burden of less than 1.5 g; similar PyMT mRNA levels in lungs was found in the GP1b^{null};PyMT (n=14) mice as compared to PyMT mRNA in control GP1b^{WT};PyMT (n=9) mice with an insignificant *p* value of 0.38 (Fig 4A). In the other group of animals which had a cumulative primary tumor burden of more than 1.5 g the PyMT mRNA levels in lungs were similar between control GP1b^{WT};PyMT (n=7) and GP1b^{null};PyMT (n=9) (*p*=0.25) animals (Fig 4B). These data suggest no significant difference in spontaneous lung metastasis between control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies and the absence of GP Ibα from the platelet surface does not influence lung metastasis in the mouse model of MMTV-PyMT.

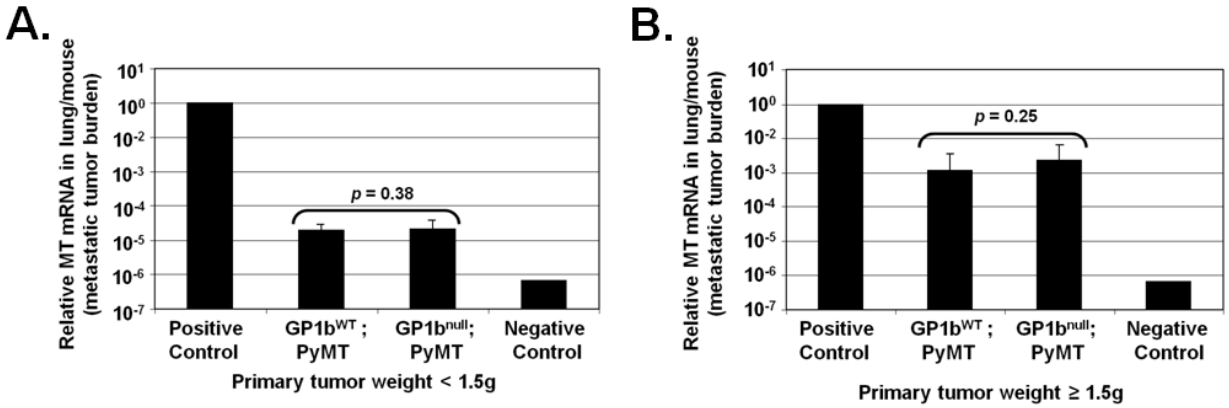

Figure 4. Lung metastatic burden in control GP1b^{WT};PyMT and GP1b^{null};PyMT mice.
*Nulligravida control GP1b^{WT};PyMT and GP1b^{null};PyMT female mice were sacrificed at the age of 20 wks. Total metastatic burden on the lung of mice which had a cumulative primary tumor weight of less than 1.5 g (**panel A**) or greater than 1.5 g (**panel B**) was determined by measuring lung PyMT mRNA using real-time PCR assay. The positive control level is the mRNA from PyMT*

induced primary tumors. The negative control in each panel is the mRNA from the lung of a wild type C57BL/6J mouse. The graph illustrates the relative lung PyMT mRNA levels in control GP1b^{WT};PyMT (n = 9) and GP1b^{null};PyMT (n = 14) mice as compared to the positive control. Error bars represent the standard deviation. p value (Student's t test) is shown.

Interpretation of Data

In the experimental metastasis assays, a 95% reduction in GP1b^{null} mice was observed, while in spontaneous lung metastasis no difference was found between control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies. Different cancer cells, immunologic response, routes of dissemination, and strain variation may be some of the reasons responsible for the different outcomes in experimental and spontaneous metastasis assays. An immortalized cell line B16F10.1 mouse melanoma cells was used in the experimental metastasis assays. Although, these cells originated from the C57BL/6J strain, since they have been grown in a two dimensional tissue culture for an indefinite time, it is possible that they have lost some of their characteristic features and acquired some additional traits which may have changed their metastatic potential. In MMTV-PyMT mice metastatic tumors are formed by freshly dislodged tumor cells which might be assumed to retain all the cellular properties of a typical cell in a natural tumor environment. Nevertheless, discrepancy in conclusions on the relevance of GP Ib-IX for metastasis does exist. A similar discrepant conclusion was found when analyzing the protease activated receptor (PAR) 1 mouse knockout in the MMTV-PyMT model, PAR 1 knockout animals have reduced experimental metastasis but no difference in metastasis driven by MMTV-PyMT [11].

In experimental metastasis the direct injection of a large number of tumor cells into the venous circulation provides an excellent opportunity for tumor cells to interact with different vascular components, such as platelets, immediately after injection. In the model of MMTV-PyMT breast cancer metastasis, a smaller number of cells presumably first enter lymphatic vessels. Subsequently, they enter the blood circulation. As a result tumor cells have a longer and different vascular path to metastasize to the lungs as compared to experimental metastasis. This too, could alter the behavior of tumor cells leading to different outcomes in spontaneous PyMT metastasis versus experimental metastasis.

On the basis of observations obtained from control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies it can be concluded that platelet GP Ib-IXis dispensable for the growth of spontaneous primary mammary adenocarcinoma and associated spontaneous lung metastasis in the MMTV-PyMT model. However, while expression of PyMT may be a better model to study degradation of stroma surrounding the primary tumor and tumor cell migration; it has not proven to be the most reliable model for studying relevance of vascular components in hematogenous metastasis due to high variability in latency and the highly tumorigenic nature of PyMT generated tumor cells [12]. Future studies will investigate the contribution of platelet GP Ibα in

a more controlled spontaneous tumorigenesis model where oncogenic activation can be induced at a precise time point.

Key Research Accomplishments as of August 2010

1. **In vivo imaging has demonstrated in models of experimental metastasis that platelet GPIb-IX does not facilitate tumor cell homing to the lung (SA1).**
2. **Histological analysis has identified a platelet/macrophage axis that is significantly altered in animals devoid of platelet GPIb-IX (SA1-SA3).**
3. **A congenic mouse colony expressing a variant of the GPIb-IX complex in which a Tyr 276 has been changed to Phe has been established after more than 10 generations of backcross mouse husbandry (SA2).**
4. **A model of spontaneous breast tumor development and metastasis has been developed in C57BL/6J mice devoid of the platelet GPIb-IX receptor. Detailed studies of primary tumor growth and metastasis have been performed.**

Reportable Outcomes

1. **Oral symposia speaker at the European Society of Hematology meeting, June 2010, talk entitled "The Platelet Paradigm in Thrombosis and Cancer"**

Conclusion

Our project tests the hypothesis that circulating blood platelets contribute to tumorigenesis with a significant impact on molecular events leading to metastatic disease. We continue to use the mouse models we developed in our laboratory to address fundamental questions related to platelet adhesion and activation in the breast cancer disease process.

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